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PRINCIPAL INVESTIGATOR: Sunshine Daddario, B.A.

CONTRACTING ORGANIZATION: University of Colorado Health Sciences Center
Aurora CO 80045-0508

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Sunshine Daddario, B.A.

E-Mail: Sunshine.Daddario@uchsc.edu

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13. SUPPLEMENTARY NOTES

14. ABSTRACT: HOX genes encode a large family of transcription factors involved in key developmental decisions, and are often aberrantly expressed in cancer. Our laboratory has previously shown that a subset of genes of the HOXC cluster are overexpressed in primary prostate tumors, metastases, and prostate cancer (PCa) cell lines1. Increasing transient expression of HOXC8 in LNCaP PCa cells as well as HPr-1 AR non-tumorigenic prostate epithelial cells results in a progressive suppression of androgen responsive promoters. Transcription from both the mouse probasin promoter and the MMTV promoter is inhibited at levels of HOXC8 expression comparable to those seen in PCa cell lines. Other members of the HOX family also inhibit androgen signaling. We have created LNCaP and HPr-1 AR derived lines that stably overexpress HOXC8 and show that signaling through androgen responsive promoters is inhibited, and PSA mRNA levels are decreased in these cell lines. HOX proteins block the histone acetyltransferase activity of the coactivators CBP and p3002. As these are key mediators of steroid-dependent transcription, inhibition of these coactivators could account for the HOX-dependent suppression of androgen receptor-mediated transcription. We show that overexpression of CBP relieves the inhibition of androgen receptor-mediated transcription by HOXC8. Further, chromatin immunoprecipitation demonstrates that HOXC8 expression inhibits hormone-induced histone acetylation at MMTV. HOXC8 overexpression has been shown to correlate with higher Gleason grade PCa3. Our preliminary studies demonstrate that stable overexpression of HOXC8 increases HPr-1 AR invasiveness in vitro. In contrast to androgens, the secosteroid vitamin D has been shown to have antiproliferative, prodifferentiation and antimetastatic properties in PCa. Increasing expression of HOXC8 also results in a progressive suppression of vitamin D-induced transcription in vitamin D-responsive ALVA-31 PCa cells. Interestingly, in LNCaP cells, overexpression of HOXC8 does the reverse. Here HOXC8 potentiates hormone-induced transcription from various vitamin D-responsive promoters. continued on next page.

15. SUBJECT TERMS
ANDROGENS, PROSTATE CANCER, HOMEOBOX PROTEINS, HOX, ANDROGEN INDEPENDENT PROSTATE CANCER

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ABSTRACT continued

These data indicate that the simple model of HOX overexpression impacting intracellular receptor activity through the inhibition of CBP/p300 is, at best, only part of the story. We propose that increased expression of HOXC genes during tumorigenesis precipitates a need for the tumor cells to overcome HOXC-mediated inhibition of androgen signaling, predisposing tumors cells to survive in the face of a subsequent androgen withdrawal. Understanding the mechanism underlying the failure of endocrine intervention could lead to the design of therapeutic modalities that would prolong the efficacy of androgen ablation therapy.

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INTRODUCTION

In the androgen-responsive, normal prostate, essentially no expression of HOXC genes is seen. Our laboratory has previously shown that a subset of genes of the HOXC cluster are overexpressed in primary prostate tumors, metastases, and PCa cell lines[2]. Although the consequences of HOXC overexpression in the prostate are unknown, we have shown that HOXC6 or HOXC8 overexpression inhibits androgen receptor (AR)-mediated transcription in LNCaP cells. The goal of this work is to explore further the interplay between HOXC expression and steroid receptor signaling, investigating both the underlying mechanisms and the consequences of HOXC overexpression on androgen action in prostate cells.

BODY

Two tasks were listed in the approved Statement of Work:

Task one: To characterize the consequences of HOXC expression on steroid signaling in human PCa cell lines.

Task two: To dissect the molecular mechanism of HOXC inhibition of androgen signaling.

Task One: To characterize the consequences of HOXC expression on steroid signaling in human PCa cell lines.

We have previously shown that HOXC8 and HOXC6 transient overexpression inhibits AR-mediated transcription of the androgen-responsive MMTV and probasin promoters in LNCaP and PC-3 AR PCa cells. We created a series of cell lines by viral transduction which stably overexpress HOXC8 and have shown that HOXC8 inhibits AR-mediated signaling when stably overexpressed in LNCaP, DU-145, PC-3-AR, and ALVA-31 cells.

In order to further characterize the consequences of HOXC expression in the context of PCa, we utilized various tumorigenicity assays in LNCaP PCa cells stably overexpressing HOXC8. These assays include cell proliferation (Hoechst staining), invasion and migration (Boyden chamber), and soft agar colony formation (anchorage-independent growth). Thus far we have been unable to detect any significant difference between the HOXC8 overexpressing cell lines and control cell lines in these assays (data not shown). Because HOXC8 overexpression may be involved in early tumorigenesis, we reasoned that it would be important to perform these tumorigenicity assays in cells lines derived from non-tumorigenic “normal” prostate epithelial cells. We therefore created cell lines overexpressing HOXC8 using RWPE-I and PWR-IE non-tumorigenic prostate epithelial derived cell lines, both of which have been reported to express AR. In our hands however, no androgen receptor protein was detected by western analysis after chronic treatment with the synthetic androgen R1881 for up to 4 weeks (data not shown), and there was no detectable induction of androgen signaling in reporter assays using the androgen responsive reporters PSA-luc, MMTV-luc and probasin-luc (data not shown). These cell lines were therefore not useful for our studies of the interaction of HOXC8 and AR signaling.

We therefore obtained another non-tumorigenic prostate epithelial derived cell line, HPr-1 AR. These cells express androgen receptor (Fig. 1A) and are induced upon androgen treatment through the probasin promoter in luciferase reporter assays (Fig. 1B). We virally transduced these cells and confirmed overexpression of HOXC8 protein levels by western analysis (Fig. 2). In reporter assays, induction of the PSA-luciferase promoter is inhibited in HPr-1 AR HOXC8 cells when compared with empty vector transduced control cells (Fig. 3). HOXC8 overexpression has been shown to correlate with higher Gleason grade PCa[3]. Our preliminary studies suggest that HPr-1 AR-HOXC8 are more invasive *in vitro* (Fig. 4). Preliminary characterization studies of HPr-1 AR HOXC8 have shown no difference in cell proliferation, cell cycle, soft agar colony formation or migration between HPr-1 AR HOXC8 cells and those transduced with empty vector.

Task two: To dissect the molecular mechanism of HOXC inhibition of androgen signaling.

Our initial findings that HOXC8 and HOXC6 overexpression inhibit AR-mediated transcription in PCa cells, coupled with reported data demonstrating that homeodomain-containing proteins interact with and inhibit the histone-acetyltransferase (HAT) activity of the steroid receptor coactivators CBP and p300[1], lead us to hypothesize that HOXC proteins inhibit AR-mediated signaling through inhibition of CBP/p300 HAT activity. In support of this hypothesis, we have demonstrated that increased expression of CBP relieves HOXC8 induced inhibition of AR-mediated transcription of the androgen-responsive MMTV promoter in a dose dependent manner in LNCaP PCa cells. We will perform similar CBP overexpression studies with the HPr-1 AR HOXC8 cell line we have recently created. Further, we have previously demonstrated by chromatin immunoprecipitation (ChIP) that hormone-induced histone acetylation (H4) at the androgen-responsive MMTV promoter is inhibited upon overexpression of HOXC8 in LNCaP PCa cells. We have confirmed that acetylation of histone H4 (as well as histone H3) are similarly inhibited in PC-3 AR PCa cells (Fig. 5). Future experiments will include ChIP analysis of the endogenous PSA promoter and enhancer regions in LNCaP and HPr-1 AR cell lines stably overexpressing HOXC8.

KEY RESEARCH ACCOMPLISHMENTS

- Development and characterization of several HOXC8 overexpressing PCa and non-transformed prostate epithelial cell lines by viral transduction
- Successful implementation of ChIP analysis using transiently transfected target DNA
- Successful siRNA knockdown of HOXC8 protein levels in LNCaP PCa cells

REPORTABLE OUTCOMES

Abstract: HOXC Gene Expression Modulates Androgen- and Vitamin D- Mediated Actions in Human Prostate Cancer Cells; IMPaCT meeting, September 5-8, 2007.

Abstract: HOXC Gene Expression Modulates Androgen- and Vitamin D- Mediated Actions in Human Prostate Cancer Cells; Nuclear Receptors: Steroid Sisters, Keystone meeting, March 30-April 4, 2008.

CONCLUSIONS

We have extended upon our initial observations demonstrating that HOXC6 and HOXC8 inhibit AR-mediated transcription in LNCaP PCa cells in transient reporter assays to include congruent data from LNCaP PCa cells as well as non-tumorigenic HPr-1 AR cells stably overexpressing HOXC8, all further demonstrating HOXC8 inhibition of AR-mediated transcription in prostate cells.

We hypothesize that HOX proteins inhibit AR-mediated signaling through inhibition of HAT activity of the steroid receptor coactivators CBP/p300. In support of this hypothesis, we have demonstrated that increased expression of CBP relieves HOXC8-induced inhibition of AR-mediated transcription in a dose dependent manner. Further, we have demonstrated by ChIP analysis that hormone-induced histone acetylation at the androgen-responsive MMTV promoter in inhibited upon overexpression of HOXC8 in both LNCaP and PC-3 AR PCa cell lines.

We have also performed various tumorigenicity assays in HOXC8 overexpressing cell lines, including cell proliferation, migration, invasion and soft agar colony formation (anchorage independent growth). We were unable to detect any significant difference between the HOXC8 overexpressing PCa cell lines and control cell lines in these experiments, which led us to believe that HOXC8 overexpression may be involved in an early step in tumorigenesis, and therefore it is critical to perform these assays in non-tumorigenic prostate epithelial cell lines. We have therefore recently created cell lines stably overexpressing HOXC8 using HPr-1 AR non-tumorigenic prostate epithelial derived cell lines. These initials studies have shown successful as we have demonstrated that signaling through the PSA promoter is inhibited, and our preliminary studies suggest that overexpression of HOXC8 in these cells leads to increased invasiveness in Boyden chamber/Matrigel *in vitro* invasion assays. We believe that further characterization and analysis of these cell lines will prove extremely informative in elucidating the role of HOXC8 in androgen receptor-mediated signaling and prostate tumorigenesis.

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(1) Shen WF, Krishnan K, Lawrence HJ, Largman C. The HOX homeodomain proteins block CBP histone acetyltransferase activity. Mol Cell Biol. 2001 Nov;21(21):7509-22.

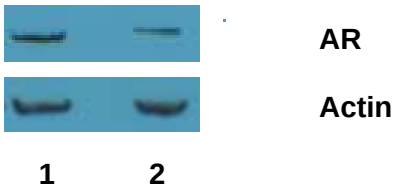
(2) Miller GJ, Miller HL, van Bokhoven A, Lambert JR, Werahera PN, Schirripa O, Lucia MS, Nordeen SK. Aberrant HOXC expression accompanies the malignant phenotype in human prostate. Cancer Res. 2003 Sep 15;63(18):5879-88.

(3) Waltregny D, Alami Y, Clausse N, de Leval J, Castranovo V. Overexpression of the Homeobox Gene HOXC8 in Human Prostate Cancer Correlates with Loss of Tumor Differentiation. The Prostate. 2002 Feb 15;50(3):162- 9.

APPENDICES- none

SUPPORTING DATA

A)



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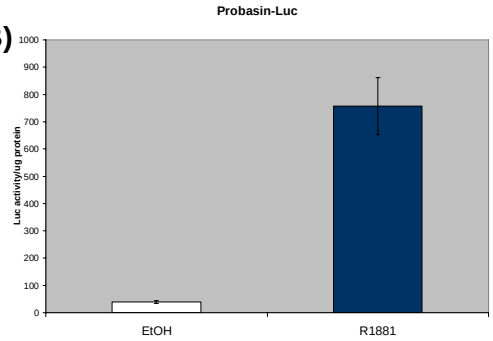


Fig. 1A. HPr-1 AR (1) express AR by western analysis. LNCaP (2) positive control.

1B. HPr-1 AR show induction through the probasin-luciferase reporter when treated with R1881 for 18 hours.

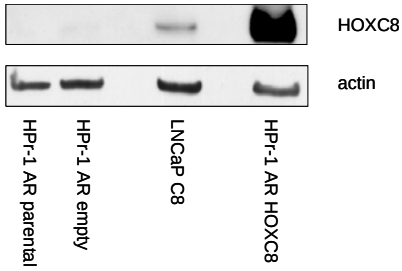


Fig. 2. HPr-1 AR HOXC8 overexpress HOXC8 protein by western analysis.

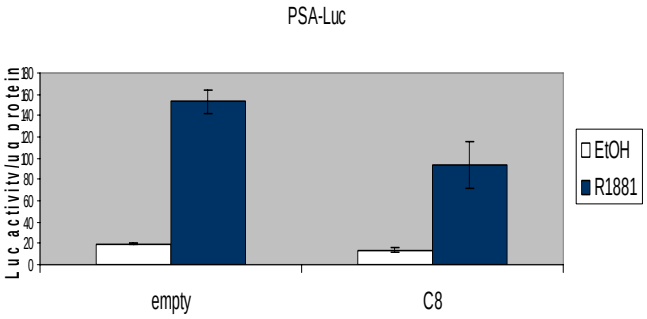
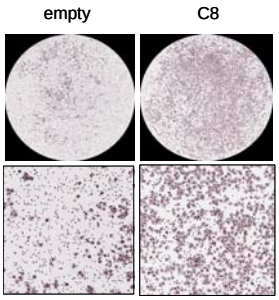


Fig. 3. Induction of signaling through the PSA promoter is inhibited in HPr-1 AR cells stably overexpressing HOXC8 in luciferase reporter assays.

A)



B)

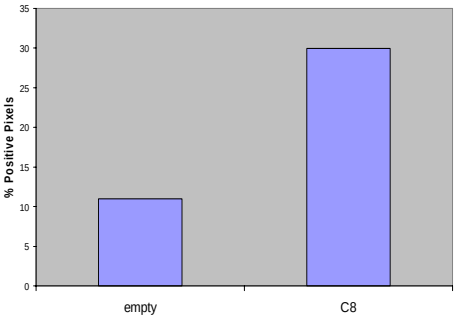


Fig. 4. HPr-1 AR HOXC8 are more invasive in vitro when compared with empty vector control HPr-1 AR cells. 3 day Matrigel Boyden chamber assay.
A) Scanned images of stained cells which have invaded through Matrigel coated filters.
B) Quantification of invading cells.

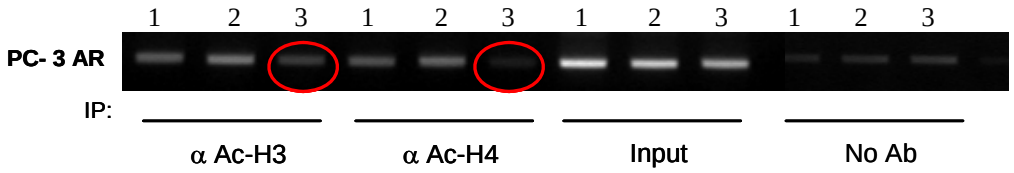


Fig. 5. Histone acetylation (H3 & H4) is inhibited by transient overexpression of HOXC8 in PC-3 AR PCa cells by chromatin immunoprecipitation. (1) EtOH, (2) 10^{-8}M R1881; (3) 10^{-8}M R1881 + 100ng/ml HOXC8. Empty vector used to balance total plasmid transfected into cells.